Genetic relationships between southern African SAT-2 isolates of foot-and-mouth-disease virus

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(Accepted 3 June 1992)

SUMMARY

Sequencing of part of the 1D gene of foot-and-mouth disease virus was used to determine the relationships between SAT-2 viruses isolated from outbreaks which occurred in cattle in Zimbabwe and Namibia and in impala in South Africa between 1979 and 1989. The results demonstrated that the outbreaks in different countries were unrelated. Surprisingly close relationships were shown between all SAT-2 viruses isolated from cattle in Zimbabwe since 1983 but the two major epizootics which occurred in 1989 were caused by viruses which were clearly different. Conversely, two apparently unrelated outbreaks in impala in South Africa were caused by viruses which could not be distinguished.

INTRODUCTION

Foot-and-mouth disease (FMD), a highly contagious disease of cloven-hoofed animals, is a serious constraint on the expansion of export-orientated agricultural activity in southern Africa. Botswana, Namibia, South Africa and Zimbabwe, which all rely on exports of animal products, are required to maintain strict control of FMD in order to preserve their markets in developed regions of the world. When serious epizootics in domestic livestock occur, as in Zimbabwe in 1989, the consequences can be disastrous due to the loss in export earnings. A projected Beef Trading Account surplus of Z$4156000 for 1989 prior to the epizootic in Zimbabwe become a predicted deficit of Z$8909000 after the outbreak [1]. The eventual total cost to Zimbabwe of the 1989 outbreaks is likely to be in the region of Z$200 million.

Tracing the origin of FMD outbreaks is essential for the effective control of FMD and has been accomplished in Europe [2]. In southern Africa, however, this is complicated by the presence of free-living maintenance hosts, principally buffalo (Syncerus caffer), the presumed usual source of infection for other species [3–5]. Due to their mobility, individual buffalo and even groups are difficult to trace and even more difficult to sample. There is no evidence that types of FMD virus other than the SAT-types are established in buffalo populations in southern Africa.
In the Kruger National Park (South Africa), infection rates with SAT-1, 2 and 3 viruses are high in buffalo, and most individuals older than one year have neutralizing antibody to all three types (unpublished data). This situation probably also applies to most other large buffalo populations in the subcontinent [3, 4, 6]. However, although clinical FMD in buffalo in the field is rare [4], there is strong circumstantial evidence that most FMD outbreaks in cattle in southern Africa are derived from buffalo although the mechanism(s) of transmission remains obscure. The role of impala (Aepyceros melampus) in the epidemiology of FMD in southern Africa remains to be determined because although antibody to FMD viruses has been detected in the sera of impala elsewhere in southern Africa, clinical disease has only been recognized in the Kruger National Park (KNP) [3, 7]. There, outbreaks in this species occurred regularly between 1967 and 1983. Since then, however, the frequency has declined.

In 1989, two geographically separate epizootics, both caused by SAT-2 viruses, occurred in Zimbabwe. Other outbreaks of FMD, also caused by SAT-2 type viruses, occurred in impala in the KNP in 1988/89, and in cattle in the Caprivi Strip (Namibia) in 1989 (Fig. 1). Apart from establishing the relationship between these outbreaks, it was important to ascertain whether the different foci of infection in Zimbabwe, Namibia and South Africa represented distinct epizootics or extensions from earlier outbreaks. We have attempted to do this by determining the relationships between FMD viral isolates by primer extension sequencing [8–11] of a portion of the 1D (VPI) gene, which codes for the major immunogenic determinant of FMDV [12–14].
### Table 1. SAT-2 virus isolates included in this study

<table>
<thead>
<tr>
<th>Virus designation</th>
<th>Species</th>
<th>Date of outbreak</th>
<th>Geographical origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>SAR 16/83†</td>
<td>Impala</td>
<td>07/07/83</td>
<td>31° 48' E, 24° 50' S Leeuapn</td>
</tr>
<tr>
<td>KNP 1/85*</td>
<td>Impala</td>
<td>21/11/85</td>
<td>31° 50' E, 24° 15' S Gudzane area</td>
</tr>
<tr>
<td>KNP 15/88†</td>
<td>Impala</td>
<td>12/10/88</td>
<td>31° 26' E, 24° 28' E Orpen gate</td>
</tr>
<tr>
<td>KNP 16/88†</td>
<td>Impala</td>
<td>10/10/88</td>
<td>31° 24' E, 24° 27' S Kingfisher spruit</td>
</tr>
<tr>
<td>KNP 17/88†</td>
<td>Impala</td>
<td>07/10/88</td>
<td>31° 24' E, 24° 28' S Orpen gate</td>
</tr>
<tr>
<td>KNP 18/88†</td>
<td>Impala</td>
<td>11/10/88</td>
<td>31° 24' E, 24° 28' S Orpen gate</td>
</tr>
<tr>
<td>KNP 19/88†</td>
<td>Impala</td>
<td>11/10/88</td>
<td>31° 30' E, 24° 27' S Rabelais dam</td>
</tr>
<tr>
<td>KNP 20/88†</td>
<td>Impala</td>
<td>25/10/88</td>
<td>— Timbavati river</td>
</tr>
<tr>
<td>KNP 2/89†</td>
<td>Impala</td>
<td>30/04/89</td>
<td>31° 43' E, 24° 13' S Ngotso</td>
</tr>
<tr>
<td>RHO 2/79‡</td>
<td>Cattle</td>
<td>02/07/79</td>
<td>— Unknown</td>
</tr>
<tr>
<td>ZIM 5/81*</td>
<td>Cattle</td>
<td>06/11/81</td>
<td>27° 30' E, 17° 55' S Manjolo TTL</td>
</tr>
<tr>
<td>ZIM 5/83‡</td>
<td>Cattle</td>
<td>14/07/83</td>
<td>28° 05' E, 19° 45' S Nyamandhlovu</td>
</tr>
<tr>
<td>ZIM 7/83‡</td>
<td>Cattle</td>
<td>14/07/83</td>
<td>28° 05' E, 19° 45' S Nyamandhlovu</td>
</tr>
<tr>
<td>ZIM 1/87*</td>
<td>Cattle</td>
<td>25/03/87</td>
<td>29° 10' E, 19° 47' S Insiza</td>
</tr>
<tr>
<td>ZIM 2/87*</td>
<td>Cattle</td>
<td>25/03/87</td>
<td>29° 00' E, 19° 45' S Insiza</td>
</tr>
<tr>
<td>ZIM 262‡</td>
<td>Cattle</td>
<td>22/04/87</td>
<td>29° 30' E, 20° 20' S Filabusi</td>
</tr>
<tr>
<td>Zim 5/87‡</td>
<td>Cattle</td>
<td>01/07/87</td>
<td>31° 30' E, 21° 00' S Triangle</td>
</tr>
<tr>
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<td>Cattle</td>
<td>01/07/87</td>
<td>31° 30' E, 21° 00' S Triangle</td>
</tr>
<tr>
<td>ZIM 8/89‡</td>
<td>Cattle</td>
<td>27/04/89</td>
<td>30° 40' E, 17° 10' S Mutorashanga</td>
</tr>
<tr>
<td>ZIM 35/89‡</td>
<td>Cattle</td>
<td>27/04/89</td>
<td>30° 40' E, 17° 10' S Mutorashanga</td>
</tr>
<tr>
<td>ZIM 9/89‡</td>
<td>Cattle</td>
<td>04/05/89</td>
<td>29° 45' E, 19° 30' S Gweru</td>
</tr>
<tr>
<td>ZIM 266‡</td>
<td>Cattle</td>
<td>04/05/89</td>
<td>29° 45' E, 19° 30' S Gweru</td>
</tr>
<tr>
<td>ZIM 12/89*</td>
<td>Cattle</td>
<td>07/05/89</td>
<td>28° 35' E, 20° 10' S Bulawayo</td>
</tr>
<tr>
<td>ZIM 13/89*</td>
<td>Cattle</td>
<td>05/05/89</td>
<td>29° 43' E, 19° 10' S Kwekwe</td>
</tr>
<tr>
<td>ZIM 14/89*</td>
<td>Cattle</td>
<td>17/05/89</td>
<td>28° 40' E, 20° 12' S Bulawayo</td>
</tr>
<tr>
<td>ZIM 16/89*</td>
<td>Cattle</td>
<td>19/07/89</td>
<td>30° 48' E, 18° 38' S Featherstone</td>
</tr>
<tr>
<td>ZIM 19/89*</td>
<td>Cattle</td>
<td>30/08/89</td>
<td>30° 30' E, 17° 40' S Darwendale</td>
</tr>
<tr>
<td>NAM 4/89‡</td>
<td>Cattle</td>
<td>22/11/89</td>
<td>25° 01' E, 17° 44' S East Caprivi</td>
</tr>
</tbody>
</table>

* Pirbright reference number.
† Onderstepoort reference number.
‡ Botswana Vaccine Institute reference number.
§ Isolates were made from the same material at different laboratories.

### MATERIALS AND METHODS

**Viruses**

The origin of the viral isolates used in the study are given in Table 1. They were isolated on primary bovine thyroid or pig kidney cells and stored in aliquots at −70 °C (stock virus). Where so indicated the same material was used to isolate virus and sequence it independently at the two laboratories involved in the investigation.

**Serology**

Virus neutralization tests (VNT) were performed as described previously [15].
Fig. 2. For legend see opposite.
Partial purification of FMDV isolated and RNA extraction

Stock viruses were passaged twice in IB-RS-2 cells; the first passage at a multiplicity of infection (m.o.i.) of approximately 0.5 and the second at a m.o.i. of approximately 10. The clarified harvest was pelleted through a 30% (w/v) sucrose cushion by high-speed ultracentrifugation and the virus RNA extracted directly from the pellet using the procedure described by Rico-Hesse and colleagues [16] for poliovirus.

Sequencing analysis

A modification [17, 18] of the dideoxy-sequencing procedure for RNA templates described by Zimmern and Kaesberg [19] was used. The sequence of the primer (dGAGUCCAACCCUGGGCCCUUC) and its location on the genome have been described [9]. The majority of sequences in this study start at codon 150 of the VP1 gene and end of codon 216. The exceptions are shown in Figs 2 and 3. Sequencing was conducted independently at the two laboratories involved. Ambiguous or undefined bands were not included in the comparisons. The dendrograms were drawn using PHYLIP version 3.1 [20].

FMD outbreaks

Localities mentioned in the text are indicated in Fig. 1 and details pertaining to specific isolates are summarized in Table 1. Isolates from the KNP are designated KNP or SAR, those from Zimbabwe, ZIM (with the exception of one designated RHO) and the Namibian isolate NAM. Features of the FMD outbreaks were as follows.

Kruger National Park. During October 1988 an outbreak of FMD caused by a SAT-2 virus occurred in impala in the Orpen Gate area of the KNP and in the adjoining Manyeleti Game Reserve. Six virus isolates were obtained from animals sampled during the outbreak (KNP 15/88–KNP 20/88; Table 1). In May 1989 another SAT-2 outbreak was detected further north along the Timbavati river near Ngotso (Fig. 1; KNP 2/89). Although no serologically positive animals could be found in the area between the two outbreaks (unpublished data), it was necessary to establish whether these were different outbreaks or constituted a single epizootic with two foci. Virus isolates from previous outbreaks in impala, which were reported at Leeupan in 1983 (SAR 16/83) and in the Gudzane area in 1985 (KNP 1/85) were also investigated for relatedness.

Zimbabwe. The location of the SAT-2 outbreak which occurred during 1979 (RHO 2/79) could not be determined with certainty. Prior to 1983, most FMD outbreaks in Zimbabwe had occurred in the FMD endemic areas confined to the lowveld of the south-east and west [21]. However, in 1983 an outbreak caused by a SAT-2 virus occurred at Nyamandhlou, near Bulawayo (Fig. 1; ZIM 5/83; Table 1). In 1987 a number of outbreaks were recorded and ZIM 1/87 and ZIM 5/83...
2/87 were both isolated from samples submitted from Blackwaters Ranch, Insiza in March. However, this was probably not the original focus. Subsequent spread was presumed to be the source of the outbreak at Tsomo Ranch, Filabusi in April (ZIM 262). The outbreak at Triangle in July (ZIM 5/87, ZIM 263) was considered by field investigators to be a totally separate outbreak.

No further outbreaks occurred until April 1989 when FMD was diagnosed at Delken Farm, Mutorashanga in the north (Fig. 1; ZIM 8/89, ZIM 35/89). It was subsequently discovered that cattle had been moved to Delken Farm from a property adjacent to Blackwaters Ranch in February 1987. Less than 2 weeks after the Mutorashanga outbreak was diagnosed (May 1989), dispersal of infected cattle from a sale at Gweru resulted in widespread outbreaks at Bulawayo, Darwendale, Featherstone and Kwekwe (Fig. 1; ZIM 9/89, ZIM 266 and ZIM 12/89, 13/89, 14/89, 16/89, 19/89).

Namibia. During November 1989 a small localized outbreak of SAT-2 occurred in cattle in the eastern Caprivi region of Namibia (Fig. 1; Table 1; NAM 4/89).

RESULTS

The nucleotide sequences determined by the two laboratories and deduced amino acid sequences are shown in Figs 2 and 3. Since viral RNA and not cloned cDNA was used as template for the sequence analysis, heterogeneous mixtures of viral mutants could have caused ambiguous results in certain positions of the nucleotide sequence. Such positions, along with those caused by premature termination of DNA synthesis due to secondary structures in the RNA template, are marked by an asterisk (*) in Fig. 2 and were ignored in the calculation of the relationships between the different isolates. Where the sequence of a virus was determined by both laboratories, only small differences, probably due to different passage histories, occurred (Fig. 2) [22].

The percentage nucleotide differences between the six impala viruses obtained from the 1988 focus in the KNP, one from the 1989 focus, and viruses previously isolated from impala in the KNP in 1983 and 1985, are shown in Figure 4. The 1989 isolate and five of those from 1988 were identical over the region of the genome sequenced, while KNP 16/88 differed from these at one nucleotide position—codon 160 (Fig. 2). This results in the substitution, in KNP 16/88, of threonine by alanine (Fig. 3). The viruses from the 1983 and 1985 outbreaks share less than 83% nucleotide identity with each other and with the 1988/89 impala isolates (Fig. 4).

The KNP and Zimbabwean isolates differ by approximately 28% from each other (results not shown). With the Zimbabwean isolates that originated from outbreaks prior to 1983 (i.e. in 1979 and 1981), the nucleotide sequences tested varied by more than 18% from all the viruses isolated in 1983 and later (Fig. 5). The 1989 isolates from Mutorashanga (ZIM 8/89, ZIM 35/89) and Gweru (ZIM 9/89, ZIM 266) differed by 8% (Fig. 5). However, ZIM 8/89, ZIM 35/89 varied by less than 4% from isolates derived from previous Zimbabwean SAT-2 outbreaks which occurred between 1983 and 1987. Virus isolates which were epidemiologically linked to the Gweru isolate (ZIM 12/89, 13/89, 14/89, 16/89 and 19/89) were likewise closely related to ZIM 9/89, ZIM 266 (Fig. 5). ZIM 5/78, ZIM 263
Fig. 3. Deduced amino acid sequences of the foot-and-mouth-disease virus isolates included in this study. The consensus sequence is shown in upper case where all the amino acid residues are the same and in the lower case where the sequences differ.

* Sequence ambiguity; -, same as consensus sequence. 1 Sequencing performed at Onderstepoort; 2 sequencing performed at Pirbright. Regions not sequenced are blank.

isolated during the outbreak at Triangle in 1987 differed by 4% from the other 1987 isolates.

The isolates could also be distinguished by examining both the nucleotide and amino acid sequences (Figs 2, 3) in more detail. Four groups were found in Zimbabwe where the codon for RHO 2/79 at position 158 (Fig. 2) was CGC, that for ZIM 5/81 was AAG, while the 1983, 1987 and the Mutorashanga isolates had AAA and the Gweru related viruses had AGG at that position. A similar distinction between these four groups from Zimbabwe could also be observed at
positions 163, 185, 189, 201, 214 and 215 (Fig. 2). Furthermore, the KNP viruses could be distinguished from the Zimbabwean isolates by different nucleotides at certain positions, for example at position 160 the Zimbabwean isolates were mainly GAG while those from the KNP were predominantly ACG. This was observed at other codon positions, e.g. 163, 164, 167, 169, 171, etc. (Fig. 2). The amino acid sequences also had this clear distinction between the four groups from Zimbabwe at position 200 (Fig. 3) and between the Zimbabwean and KNP viruses at position 160, 169, 196 and 207. At both positions 196 and 207, RHO 2/79 and ZIM 5/81 had the same amino acids as the KNP isolates (Fig. 3).

The isolate made from the SAT-2 outbreak in Namibia (NAM 4/89) showed, on average, 22% difference from the KNP isolates (Fig. 4) and 25% from the Zimbabwean viruses (results not shown) and could also be distinguished from both region's isolates by unique substitutions at both the nucleotide (156, 160, 170, 176, 181, etc.) and amino acid levels (156, 160, 174, 176, 198, 200) (Figs 2, 3).

DISCUSSION

The viruses examined in this study were not plaque purified as the predominant component of a single virus isolate determines the immunogenic effect, and its sequence is more useful than that of randomly selected clones [9]. Thus heterogeneous mixtures of viral mutants could have caused the ambiguous results
encountered in some positions of the nucleotide sequence [9]. Secondary structure in the RNA could also lead to such ambiguities [23] especially where closely related viruses have bands in all four lanes at the same position in the sequence such as the KNP isolates from 1988/9 where this occurs at the middle position of codon 162.

The aligned VP1 nucleotide sequences of SAT virus types differ on average by 50% from the Eurasian types and SAT-1, -2 and -3 differ by approximately 43% from each other (results not shown). To obtain quantitative measures of all interrelationships in this study, pairwise comparisons of percentage differences between all sequences were performed and the binary trees were derived using the KITSCH program of the PHYLIP package [20]. The SAT-2 isolates from Zimbabwe and the KNP differed on average by 28% with respect to the nucleotide sequences in the region of the genome sequenced (results not shown). The isolate from Namibia differed by approximately 22% (Fig. 4) and 25% (results not shown) from the KNP and Zimbabwean isolates respectively. Geographic clustering of related FMD viruses was evident from direct visual comparison of the aligned sequences (see results) and it was concluded that the outbreaks of FMD in Zimbabwe, South Africa and Namibia were caused by distinct SAT-2 viruses. In fact, the isolates from these three countries differed so widely from each other that it may be possible to determine the probable origin of SAT-2 outbreaks in southern Africa by their relationships to these viruses.

These results indicate that there are greater differences between SAT-2 isolates than between strains within types A, O and C of FMDV [9, 11, 23–26]. However, within geographic areas, isolates are more closely related and each geographic location appears to have its own viruses evolving separately. The evolution of poliovirus (also a picornavirus) studied using RNA sequencing of several wild-type isolates across a 200 base region at the VP1-2A boundary [16], indicated that isolates from the same geographical location clustered into groups of closely related strains, the place of isolation being more important than the year. The geographic origins of outbreaks could thus be ascertained.

Although only a part of VP1 was examined in this study, certain areas of high conservation could be identified. From amino acid 176 to 195 and again from 208 to the end of VP1 (217), very few changes were observed between the SAT-2 isolates. The sequence RMKRAELYCPRPLL (181–194) is virtually unchanged between all the FMDV serotypes, while the sequence CPRP is conserved between all the picornaviruses [27]. All the SAT-2 isolates included in this study had this conserved sequence except Zim 5/81 which had alanine at position 192 instead of proline.

Because the isolates from the two outbreaks in impala in the KNP during 1988/9 were indistinguishable on the basis of the sequences examined (Fig. 4), the most obvious conclusion would be that they were derived from a single outbreak with two foci. Impala are distributed predominantly along river courses and it is possible that the virus was spread by a limited number of animals infecting each other along the Timbavati river but which were not detected in the group of impala sampled. Alternatively, it is possible that some undetermined vector enabled the virus to cross the area between the foci without infecting animals in the intervening countryside.
Two isolates (KNP 1/85 and SAR 16/83), derived from impala outbreaks which occurred in the KNP in 1983 and 1985, differ by approximately 17% from each other and by nearly 20% from the 1988/9 outbreak isolates (Fig. 4). This is probably due to the high degree of variation observed among the SAT-2 viruses isolated from carrier buffalo in the KNP, the presumed source of infection for impala. This complicates the determination of possible origins of outbreaks because percentage nucleotide differences of up to 20% have been found between buffalo isolates derived from herds within the same general locality of the outbreaks in impala (to be published).

All the viruses isolated from cattle in Zimbabwe between 1983 and 1989 form a related group with sequence differences ranging from 0 to 8% (Fig. 5). In contrast, two sequences from isolates made prior to 1983, namely ZIM 5/81 and RHO 2/79, were markedly different (17–22%) when compared with 1983–9 viruses (Fig. 5). For polioviruses, according to Rico-Hesse and colleagues [16], nucleotide differences of ≤ 5% between pairs of viruses indicate a close relationship while groups of viruses with differences ≤ 15% can be considered a ‘genotype’ and probably epidemiologically linked. Apparent relationships between viruses which show greater than 15% divergence should be regarded with caution [16]. On this basis, all the FMD virus isolates from cattle in Zimbabwe since 1983 comprise a single genotype.

Among the 7 isolates made from Zimbabwe in 1989, 5 (ZIM 12/89, 13/89, 14/89, 16/89 and 19/89) which were linked by field investigation to the dispersal sale at Gweru were closely related to the original Gweru isolates (ZIM 9/89, ZIM 266), the heterogeneity being less than 2% (Fig. 5). Thus the sequencing data confirms that obtained by field investigation. On the other hand, the Mutorashanga isolates differed from the Gweru-associated isolates by an average of 8% (Fig. 5), suggesting that the Gweru and Mutorashanga outbreak viruses were not directly linked despite being within the same genotype and having been isolated within 2 weeks of each other.

The Mutorashanga isolates (ZIM 8/89, ZIM 35/89) bore surprising homology to viruses isolated in 1987 at Triangle (ZIM 5/87, ZIM 263), Insiza (ZIM 1/87, ZIM 2/87) and Filabusi (ZIM 262) (Fig. 5). These close relationships suggest that the Mutorashanga outbreak was derived, directly or indirectly, from the previous outbreaks. This could be explained by carrier cattle maintaining the virus. Gebauer and colleagues [28] showed that during persistent infection in cattle with type C3, the VP1 gene of each isolate differed from that of the corresponding parental virus in 0–4–2–5% of positions after persistence of up to 469 days, figures which correspond closely with those found in this study. Alternatively, the 1987 outbreaks and that at Mutorashanga in 1989 may have had a common, but unknown origin. Further studies to establish the role of carrier cattle in the Zimbabwe outbreaks are in progress.

ACKNOWLEDGEMENTS

We would like to thank Dr S. K. Hargreaves, Director of Veterinary Services, Zimbabwe for helpful discussions, the Botswana Vaccine Institute for supplying the virus samples of the outbreaks in Zimbabwe and personnel of the Directorate
of Animal Health based in the Kruger National Park for supplying the impala outbreak viruses. Our sincere thanks to the University of Pretoria for the graphic work. We would also like to thank Mr P. W. Oosthuizen for excellent technical assistance.

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